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Biodegradation of the Organophosphate Insecticide Coumaphos in Highly Contaminated Soils and in Liquid Wastes

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Abstract: Approximately 400 000 litres of cattle dip wastes containing approximately 1500 mg litre^{-1} of the organophosphate insecticide coumaphos are generated yearly along the Mexican border from a USDA program designed to control disease-carrying cattle ticks. Use of unlined evaporation pits for the disposal of these wastes has resulted in highly contaminated soils underlying these sites. Previous work has shown that microbial consortia present in selected dip wastes can be induced to mineralize coumaphos. Our results demonstrate that similar microbial consortia are present in coumaphos-contaminated soils from eight waste sites and that these organisms are capable of mineralizing coumaphos in these soils using soil slurries to less than 1 mg litre^{-1} in 7–10 days at 28°C. In addition, our results show that these consortia are able to colonize pea gravel in trickling gravel filters and can be used in these filters to metabolize coumaphos from dip wastes to less than 0·1 mg litre^{-1} in 7–10 days at 28°C. These simple systems offer potential low cost means to detoxify coumaphos-containing wastes and to bioremediate soils contaminated with this organophosphate compound.

Key words: pesticide, coumaphos, bioremediation, wastewater, ticks, biofilter

1 INTRODUCTION

The Veterinary Services Branch of the US Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) runs a Tick Eradication Program designed to prevent the re-introduction of Cattle Fever into the United States through ticks on cattle imported from Mexico, or on cattle in areas of the US where there is likely exposure to ticks from Mexico. These ticks (*Boophilus annulatus* (Say) [cattle fever tick] and *Boophilus microplus* Can. [southern cattle tick]) have been eradicated from the US and re-introduction is prevented by a strict quarantine program operated along the border between the US and Mexico.

The primary tool used in the eradication program is a series of dipping vats placed at border crossing points. Cattle coming in from Mexico must be dipped in vats on the Mexican side of the border before they can enter the US. American cattle from within the quarantine zone in south Texas must be dipped in vats on the US side of the border operated jointly by the State of Texas, APHIS and the border counties, before leaving the quarantine zone. The cattle are induced to jump into the deep end of the vat resulting in their complete immersion in acaricide. They then swim the length of the vat and climb out the shallow end and remain on a drip-pad to dry. Excess pesticide that drips from the cows is channeled back into the vat. Currently, the pesticide of choice for this application is the organophosphate compound coumaphos (*O*-3-chloro-4-methyl-2-oxo-2*H*-chromen-7-yl *O*,*O*-diethyl phosphorothioate; 'Co-Ral'). The operation on the US side of the border currently employs 42 vats each containing about 15 000 litres of coumaphos at a level of about 2000 mg litre^{-1}. Vats are cleaned and recharged every two years unless limitations on the number of cattle allowed to be

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dipped, the accumulation of sediments, or the accumulation of potasan—formed by the reductive dechlorination of coumaphos under anaerobic conditions (Fig. 1)—forces more frequent recharging. These operations generate approximately 460000 litres of high-level organophosphate insecticide wastes from the operations in the US alone. The exact amount of waste generated in Mexico is unknown, but it is likely to be far greater than that generated in the United States.

Coumaphos-containing dip waste is an excellent candidate for disposal by biodegradation since these wastes are concentrated, contained, and have no other significant toxic components. Initial studies demonstrated that coumaphos could be destroyed using resting cells of parathion-hydrolase-producing *Flavobacterium* sp. followed by UV-ozonation of the hydrolysed material.[1] Parathion hydrolase is an enzyme that hydrolyses several *O,O*-diethylphosphorothioate insecticides, of which coumaphos is one. Although both coumaphos and its hydrolysis product, chlorferon, were destroyed by this combined treatment, the bulk of the carbon remained in solution, the major products being acetophenone and short-chain fatty acids. When this material was applied to soil it was quickly converted to carbon dioxide by micro-organisms present in the soil. A successful field trial was conducted at a cattle-dipping site in Laredo, Texas in which waste cattle dip was treated by amending it with xylose, ammonium sulfate and potassium phosphate and inoculating with actively growing *Flavobacterium* culture.[2,3]

More recent work by Shelton *et al.* has shown that microbial consortia present in selected vats can be induced to mineralize coumaphos.[4–8] Several organisms were isolated from enrichment cultures capable of metabolizing coumaphos. One of these, designated strain B-1, was capable of growth on coumaphos, but was inhibited by the accumulation of metabolites due apparently to the inefficient metabolism of a chlorinated intermediate(s).[9] Combination of B-1 with another organism from the vats, designated strain B-4, which metabolized these inhibitory products, yielded a stable two-member consortium that was capable of growth at the expense of coumaphos[10] (Fig. 1). Shelton and Hapeman-Somich[10] have demonstrated that these microbial consortia (with the addition of organic and inorganic nutrients) may be used to dispose of coumaphos waste solutions from a variety of cattle-dipping vats with proper aeration and pH control.

Until recently, the reductive dechlorination of coumaphos to potasan has been controlled in the field by the use of copper(II) sulfate as a biocidal agent. However, Karns *et al.*[11] have recently shown that main-

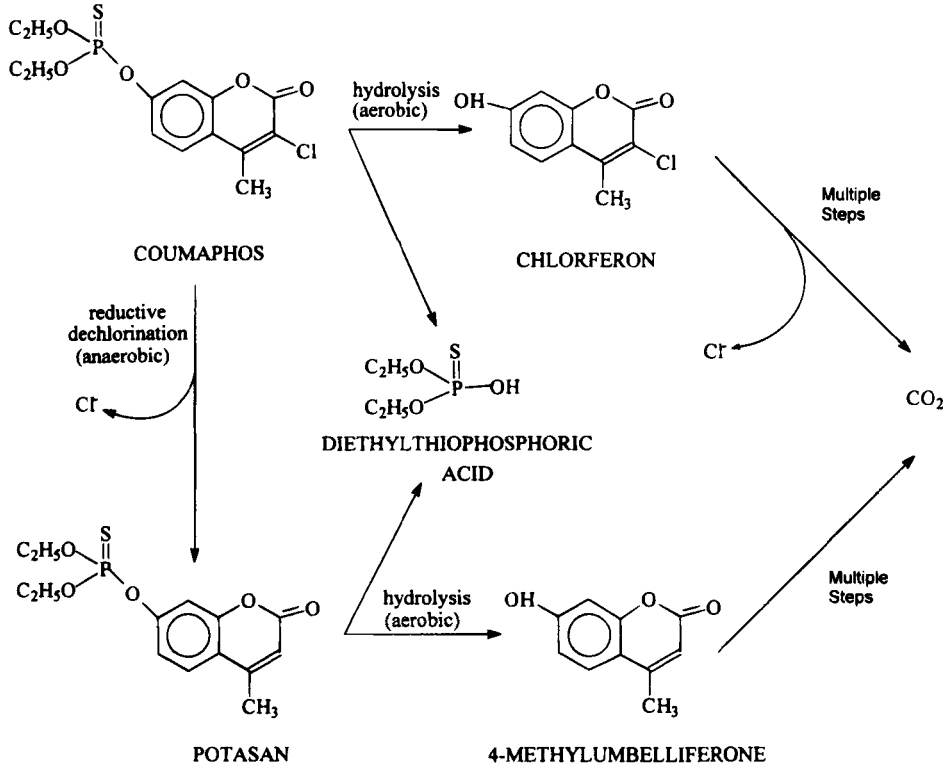

Fig. 1. Partial pathway of coumaphos metabolism under aerobic and anaerobic conditions.[5] Diethyl thiophosphoric acid and chlorferon are coumaphos hydrolysis products produced under aerobic conditions by parathion-hydrolase-producing bacteria. Potasan is produced as a result of the reductive dechlorination of coumaphos under anaerobic conditions. Aerobic hydrolysis of potasan by parathion-hydrolase-producing bacteria yields 4-methylumbelliferone and diethyl thiophosphoric acid. Chlorferon, diethyl thiophosphoric acid and 4-methylumbelliferone can be mineralized aerobically by microbial consortia present in some dip vats.

taining coumaphos suspensions at acid pH prevents the reductive dechlorination of coumaphos and leaves a suspension that is amenable to disposal by biodegradation. A field trial on an 11 000-litre coumaphos dipping vat has demonstrated the applicability of using acid to extend the life of coumaphos solutions and of biodegradation for the disposal of spent material.[11]

Currently, spent coumaphos dip is pumped into evaporation pits where the liquid leaches and/or evaporates. Since many of the evaporation pits are unlined, the underlying soils are contaminated with high concentrations of coumaphos and its metabolites. Given the success of the biodegradation of coumaphos-containing dip wastes with indigenous organisms, we sought to determine whether this strategy would also be effective for the remediation of coumaphos-contaminated soils. In addition, we devised a simple system using trickling biofilters for treating coumaphos-containing dip wastes.

2 EXPERIMENTAL METHODS

2.1 Chemicals

Analytical grade (99·6%) and formulated (42%) coumaphos, [*benzo-U*-^{14}C]coumaphos (sp. act. 21·1 mCi mmol^{-1}), potasan (94%) and chlorferon (97%) were gifts from the Animal Health Division, Bayer Corporation, Merriam, KS 66201. The [^{14}C]coumaphos was purified (99·5%) by thin-layer chromatography.[1] 4-Methylumbelliferone (97%) was purchased from Aldrich.

2.2 Sampling sites and collection

Soil samples (approximately 1 kg each) were collected from eight vat-waste disposal pits that are located along a 330-km section of Texas near the Mexican border. During sampling we attempted to include material from several sites within each pit. Material was collected from the soil surface (which was typically covered with dried plant material) down to approximately 15 cm. After shipment of the samples back to the laboratory, each soil was homogenized by hand and stored in air-tight plastic bags at 4°C. Soil analysis was performed by the Cooperative Extension Service of the University of Maryland at College Park. Table 1 provides each soil's characteristics. Coumaphos dip waste was generously provided by Elmer-Ahrens (ARS Cattle Tick Fever Research Lab, Mission, TX) and stored at 4°C.

2.3 Soil slurry incubations

Soil samples (25 g) were mixed with potassium phosphate buffer (75 ml) in 250-ml flasks and incubated at 28°C on a rotary shaker at 120 rev. min^{-1} for 7–50 days. All experiments used 50 mM potassium phosphate at pH 7·0 unless otherwise noted. Over the course of the incubations, soil slurry pH was maintained at pH 7·0 with 14·8 M ammonium hydroxide. For samples that were spiked with additional coumaphos, 0·2 g of coumaphos 420 g litre^{-1} sc ('Co-Ral'), were added per 25 g of soil. For samples that were inoculated with additional microorganisms, 5 ml of an active dip vat waste culture[11] was added per 25 g of soil. For soil washing experiments, 25-g soil samples were shaken with 1 litre distilled water, followed by centrifugation (6000*g*, 20 min, 10°C) to recover the soils. This process was repeated twice (for a total of three washes).

TABLE 1
Characteristics of Soil Samples from Eight Vat Waste Disposal Pits

Soil sample	*Soil texture*	*pH*	*CEC*[a] *(meq)*	*Organic matter (%)*
Abrams	Clay	6·8	39·1	10·8
Falcon	Organic matter[b]	6·3	39·3	21·8
Laredo	Clay-Loam	6·2	41·8	13·5
Lopena	Loam	6·8	35·6	7·5
Roma	Sandy-Clay	7·3	38·4	3·3
San Andreas	Sandy-Loam	6·4	41·2	3·7
San Ignacio	Loam	6·0	37·3	14·1
Zapata	Silt	5·6	34·5	7·0

[a] Cation exchange capacity.
[b] This sample contained a small sand fraction combined with abundant dried plant material.

2.4 Biofilter columns

Biofilter columns consisted of 30-cm lengths of 10-cm PVC pipe that were capped on the bottom and filled with either sand, pea gravel or Celite (diatomaceous earth). Coumaphos solutions were continuously pumped (at approx. 2 litre min^{-1}) from a reservoir to the top of the column using a small submersible garden pump (Beckett model M60A, Dallas, TX). A screened drain at the bottom of the column allowed the solution to flow by gravity back into the reservoir. Pea gravel used for the biofilters was obtained from a local lumber yard, Ottawa sand (20–30 mesh) was from Fisher Scientific, and Celite (Celite Biocatalyst Carrier R-635) was from Manville Sales Corp (Lompoc, CA). Coumaphos dip waste used on the biofilters was amended with potassium phosphate to a final concentration of 50 mM and brought to pH 8·0 using 10 M potassium hydroxide. Columns were seeded with microorganisms by adding 5 g of soil from the Zapata vat site.

2.5 Analytical methods

For determination of coumaphos, potasan, chlorferon and 4-methylumbelliferone, duplicate 0·1-ml samples of

soil slurries or biofilter liquids were diluted with methanol (0·9 ml), vortexed for 20 s and centrifuged for 3 min at 12 000*g*. The supernatants were analyzed by high performance liquid chromatography using a Waters C-18 Nova-Pak column with a mobile phase of methanol + 0·75 mM phosphoric acid (80 + 20 by volume).[5] The limit of detection for coumaphos was 1 mg litre^{-1} in the soil slurry experiments. In the biofilter experiments this detection limit was lowered to 0·005 mg litre^{-1} by extracting 100-ml samples of coumaphos-containing solutions with equal volumes of ethyl acetate, drying the ethyl acetate fractions, and resuspending the nearly dry residue with 5 ml of methanol. Evolution of [^{14}C]carbon dioxide was quantified by liquid scintillation.[5]

3 RESULTS

3.1 Soil slurry experiments

Soils from eight separate vat waste disposal pits were examined with respect to coumaphos degradation in 30% (w/w) soil slurries. The coumaphos concentration in the soil samples ranged from 150 to 24 000 mg litre^{-1}. As demonstrated in Fig. 2 (panel A), rapid coumaphos degradation occurred in six of the eight soil slurries. The rates of degradation were roughly similar for all six slurries, but the rates generally increased as initial coumaphos concentration increased. No aromatic metabolites were detected. Inoculation of these slurries with active coumaphos-degrading cultures yielded rates of coumaphos degradation comparable to uninoculated slurries (Panel B). In addition, soils spiked with additional formulated coumaphos (approximately 100 mg AI litre^{-1}) displayed rates of degradation similar to unspiked soils (Panel B). These results show that indigenous microbial consortia capable of degrading coumaphos are present in these soils and that the 'weathered' or 'aged' coumaphos in contaminated soils is accessible for biodegradation. In a separate experiment, [^{14}C]coumaphos was added to soil slurries containing 1200 mg litre^{-1} coumaphos. In that experiment 61% of the ^{14}C from the ring of the labeled coumaphos was recovered as [^{14}C]carbon dioxide, indicating mineralization of the aromatic portion of the coumaphos molecule (Fig. 3). In contrast, a soil slurry to which the biocide sodium azide was added showed neither loss of coumaphos nor production of [^{14}C]carbon dioxide.

3.2 Slurry experiments with Falcon and Lopena soils

In experiments using Falcon and Lopena soils, significant rates of coumaphos degradation occurred only after a long lag period (40 days) (Fig. 4, Panel A). This phenomenon did not appear to be the result of low initial numbers of degrading organisms in the soils nor

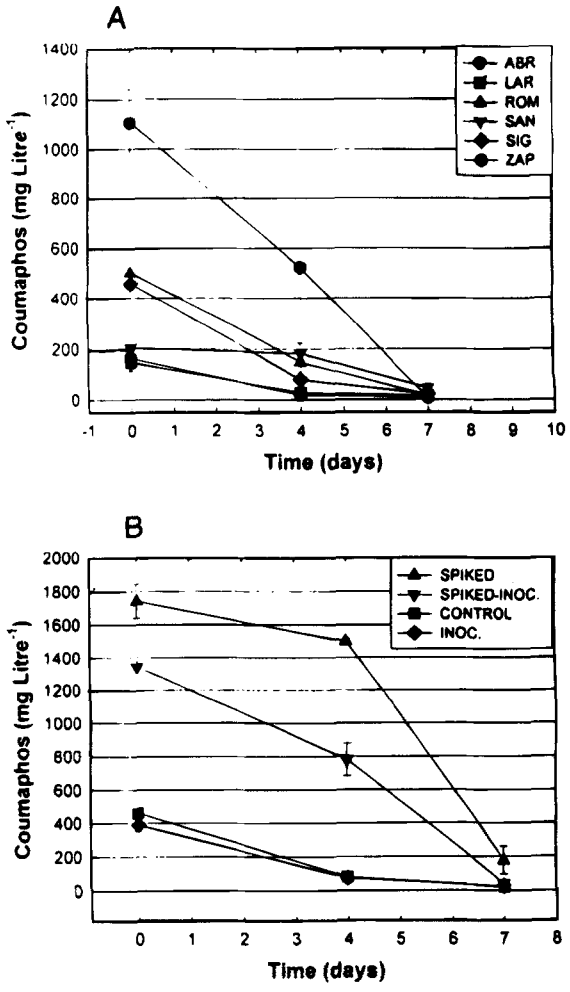

Fig. 2. Degradation of coumaphos from contaminated soils from dip vat disposal pits. Panel A shows the results of soil slurry experiments performed using individual soils from six vat sites with no amendments. Soil samples were collected from disposal pits at the following APHIS vats in Texas; ABR, Abrams; LAR, Laredo; ROM, Roma; SAN, San Andreas; SIG, San Ignacio; ZAP, Zapata. Panel B shows the results of soil slurry experiments in which flasks containing San Ignacio soil were amended with approximately 1000 mg AI litre^{-1} formulated coumaphos (spiked), amended with a 10% inoculum containing a consortium of coumaphos-degrading organisms (inoc), amended with both additional coumaphos and coumaphos-degrading organisms (spiked-inoc), or used without amendment (control). Identical experiments in which the five other soils shown in Panel A were used in soil slurries with or without coumaphos amendment or inoculation yielded similar results to those using San Ignacio soil. Error bars denote the standard deviation of coumaphos determinations using duplicate samples taken at each time point.

to non-bioavailable coumaphos, since duplicate flasks that were either inoculated with additional coumaphos-degrading organisms or spiked with fresh coumaphos showed similar patterns of degradation. Moreover, since coumaphos degradation was observed in these flasks after the lag, this phenomenon was not due the presence of high levels of non-metabolizable inhibitors (such as inorganic salts) nor to the lack of an essential nutrient.

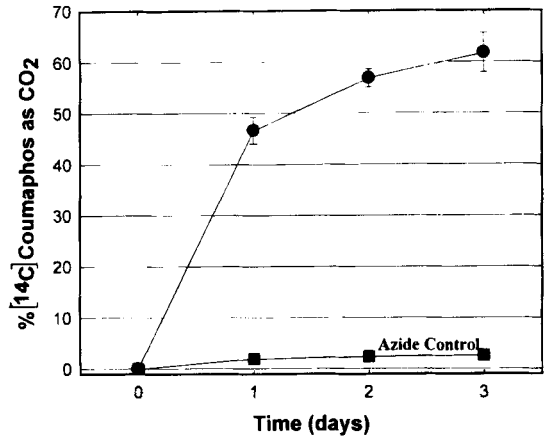

Fig. 3. Production of [^{14}C]carbon dioxide from [*benzo-U-*^{14}C] coumaphos using soil slurries containing Zapata soil. Error bars denote the standard deviation of determinations using duplicate samples taken at each time point.

Several experiments were subsequently performed to determine whether the lag period was due to the presence of a water-soluble inhibitor or to an inhibitor whose levels could be mitigated by the dilution of these soils with other soils. In the first experiment, extraction of Falcon and Lopena soils with water prior to incubation decreased the lag period preceding degradation to 7–10 days (panel B). In the second experiment, dilution of these soils with equal parts of a soil from another disposal pit (Zapata) led to the elimination of the lag period (panel C). However, soil slurries containing Lopena or Falcon soils with much less Zapata soil (using soil ratios of approximately 10 : 1) did not decrease the lag period (data not shown). One possible explanation for these results could be the presence of one or more slightly soluble inhibitory organic compounds in these soils. These compounds could be

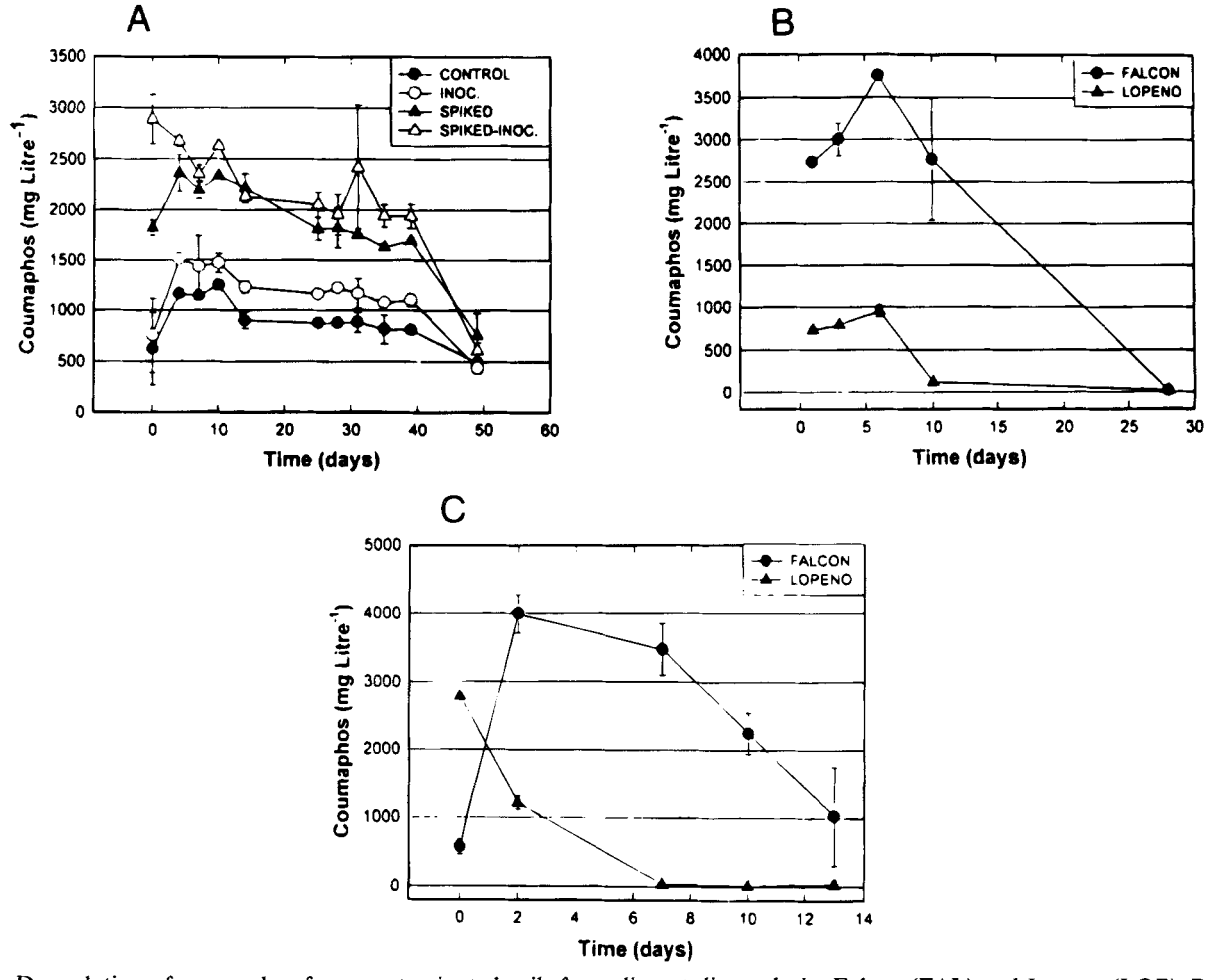

Fig. 4. Degradation of coumaphos from contaminated soils from dip vat disposal pits Falcon (FAL) and Lopena (LOP). Panel A shows the results of soil slurry experiments in which flasks containing Lopeno soil were amended with approximately 1000 mg litre^{-1} formulated coumaphos (spiked), amended with a 10% inoculum containing a consortium of coumaphos-degrading organisms (inoc), amended with both additional coumaphos and coumaphos-degrading organisms (spiked-inoc), or used without amendment (control). Identical experiments using amended and non-amended Falcon soil slurries yielded similar results. Soil slurry experiments were also performed using Falcon and Lopena soils that had been extensively washed with distilled water (panel B), or mixed with equal parts of Zapata soil (panel C). In each case, we speculate that erroneous determinations of coumaphos levels occurred in early (0–3 day) samples because a significant fraction of the coumaphos in these soils was initially associated with large particles that were excluded during sampling and only slowly broke apart. Error bars denote the standard deviation of coumaphos determinations using duplicate samples taken at each time point.

unidentified metabolites of coumaphos or could be components of the pesticide formulation. Examination of differences in the age and use of the vat sites or in the soil characteristics of each sample may lead to an understanding of why these compounds are not present at inhibitory concentrations in other samples.

3.3 Biofilter experiments

Coumaphos was rapidly removed from dip vat waste using biofilters containing sand, gravel or Celite (Fig. 5). Coumaphos levels in each of the biofilter-treated liquids decreased from 1200 mg litre^{-1} to between 0·010 and 0·050 mg litre^{-1} after 7–10 days at 25°C. No aromatic metabolites were detected by HPLC. Since coumaphos is in the form of 2-μM particles suspended in the waste, abiotic filtration of these particles by the biofilters' materials was examined using a biofilter that was treated with the biocide sodium azide. In this experiment, duplicate gravel biofilters were run in parallel for 7 days using dip waste with or without the addition of 1 mM sodium azide. The results (Fig. 6 and Table 2) show that, although coumaphos particles were effectively (>99%) filtered out by the azide-killed biofilter, coumaphos levels decreased faster and to a much lower level in the control biofilter than in the azide-treated biofilter. Extraction of residual coumaphos from these two biofilters revealed that 80% of the original coumaphos could be recovered from the azide-treated biofilter (Table 2). In contrast, less than 6% of the original coumaphos could be recovered from the control biofilter. These results demonstrate that the rapid disappearance of coumaphos from the control biofilters is accompanied by its biological degradation.

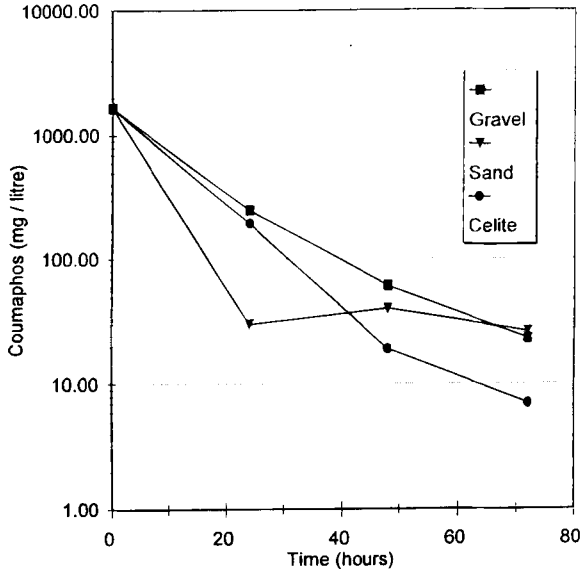

Fig. 5. Degradation of coumaphos in dip waste using biofilters. Waste dip was buffered to pH 8 with 50 mM potassium phosphate and continuously circulated through biofilters containing pea gravel, Ottawa sand or Celite pellets. A small amount (10 g) of Zapata soil was added to seed each biofilter with the microbial consortia needed for coumaphos degradation.

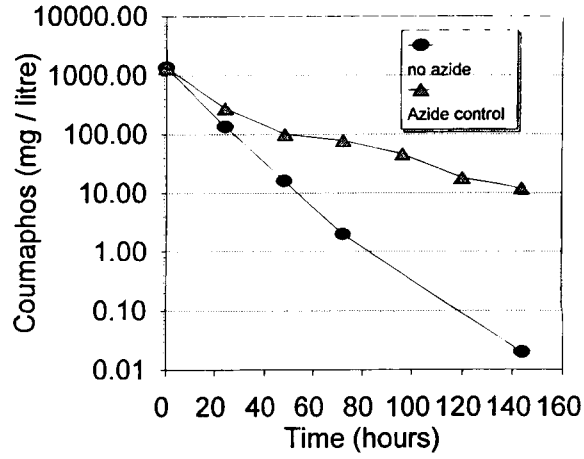

Fig. 6. Degradation of coumaphos from dip waste using a gravel biofilter. Buffered dip solutions with or without the biocide sodium azide were circulated through gravel biofilters to determine the rate and extent of coumaphos removal in biofilters due to nonbiological processes.

4 CONCLUSIONS

Our results demonstrate that biodegradation treatments using buffered soil slurries and indigenous microbial consortia are capable of rapidly mineralizing coumaphos and its metabolites from six of eight soil samples from disposal pits. Additional experiments designed to determine the range of conditions necessary for rapid coumaphos biodegradation suggest that pH 7·5–8·5 is optimal and that no additional nutrients or trace metals are necessary (data not shown). Preliminary experiments suggest that coumaphos degradation

TABLE 2
Recovery of Coumaphos from Gravel Biofilters Used to Treat Dip Waste[a]

	Azide-treated biofilter (mg litre^{-1})	*Untreated biofilter (mg litre^{-1})*
Aqueous	12	0·02
Gravel	620	54
Gravel wash	448	26
Total recovery	1080/1358	80/1358
Recovery (%)	79·5	5·9

[a] Biofilters used with dip waste (with or without sodium azide) were disassembled after 140 h (see Fig. 6). Biofilter gravel was washed with water and then extracted with methanol to recover residual coumaphos. Coumaphos levels in the dip waste (aqueous), extracted gravel (gravel) and water wash of the gravel (gravel wash) were determined.

is accelerated in moist buffered soils. However, this treatment is much slower and may fail to reach treatment targets during acceptable incubation times.

Our results show that trickling gravel biofilters colonized with coumaphos-degrading consortia quickly degrade coumaphos in waste dip solutions. Laboratory scale biofilters achieved final coumaphos levels of 0·02–0·10 mg litre^{-1} after 7–10 days using waste dip containing 1200 mg litre^{-1} coumaphos. Presently, a mobile field-scale biofilter capable of treating 15 000-litre batches of dip waste is being tested on site in Texas.

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